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Oral *N*-acetylcysteine attenuates the rat pulmonary inflammatory response to antigen

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ABSTRACT: Oxidative stress is involved in the pathophysiology of inflammatory airway diseases including asthma; therefore, antioxidants might be of clinical benefit in asthma treatment. In the present study, the effects of *N*-acetylcysteine on sensitised brown Norway rats were examined.

***N*-Acetylcysteine (3 mmol·kg body weight^{-1} administered orally) was given daily for 1 week before challenge and various antigen-induced pulmonary responses were studied.**

Antigen exposure increased lipid peroxidation in bronchoalveolar lavage fluid (BALF) and oxidised glutathione levels in lung tissue 2 h after challenge. Lung nuclear transcription factor-κB-binding activity was increased 2 h after challenge, and BALF tumour necrosis factor-α and inducible nitric oxide synthase expression in lungs peaked 4 h after challenge. Expression of intercellular adhesion molecule-1 and mucin MUC5AC was also increased 4 h after challenge. These changes in oxidant status, transcription factor activation, and inflammatory cytokine and gene expression were reduced by *N*-acetylcysteine. This thiol did not affect the immediate bronchospasm reaction to antigen in anaesthetised rats but inhibited airways hyperresponsiveness to 5-hydroxytryptamine and the augmented eosinophil numbers in BALF, which appear 24 h after exposure of conscious rats to antigen aerosol, and abolished antigen-induced extravasation of Evans blue into BALF.

These results indicate that oral *N*-acetylcysteine exerts an antioxidant protective effect and attenuates pulmonary inflammation in experimental asthma.

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Increasing clinical, epidemiological and experimental evidence indicates that excess production of reactive oxygen and nitrogen species and defective endogenous antioxidant defence mechanisms may be present in asthma [1]. Therefore, antioxidant therapy might be of clinical benefit in asthma treatment.

Although initially used as mucolytic, *N*-acetyl-L-cysteine (NAC) is a thiol compound that acts directly as a free radical scavenger and as a precursor of reduced glutathione (GSH) [2]. In contrast with the activity reported in various inflammatory pulmonary diseases, the influence of NAC in clinical asthma remains uncertain [3, 4] but has not been evaluated recently. Experimental studies demonstrate the capacity of NAC to inhibit various inflammatory elements related to oxidant stress and involved in the pathophysiology of asthma, such as nuclear factor (NF)-κB, tumour necrosis factor (TNF)-α, inducible nitric oxide synthase (iNOS) and cell adhesion molecules [5–8]. In addition, oxidative stress stimulates mucin MUC5AC synthesis in airways, a process also inhibited by NAC [9]. It has been reported recently that a relatively low dose of NAC (oral administration of 1 mmol·kg body weight^{-1}·day^{-1} for 1 week before antigen challenge) reduces airways hyperresponsiveness to 5-hydroxytryptamine (5-HT) and the augmented eosinophil numbers and Evans blue extravasation in bronchoalveolar lavage fluid (BALF) elicited by antigen exposure in actively sensitised brown Norway rats [10].

The aim of the present work was to explore further the antihyperresponsiveness and anti-inflammatory effects of orally administered NAC but using a higher dose (3 mmol·kg body weight^{-1}) for better assessment of the effects [5, 11], and to extend the study to examine lung oxidant status and expression of NF-κB, TNF-α, iNOS, intercellular adhesion molecule (ICAM)-1 and mucin MUC5AC in this model of allergic asthma.

Materials and methods

Animal model and experimental groups

Male brown Norway rats (250–300 g) were supplied by B&K Universal (Barcelona, Spain), kept at an ambient

temperature of 22°C under a 12-h phase light/dark cycle and fed on A04 pellets (Panlab, Barcelona, Spain). Drinking water was freely available. The experimental protocols were approved by the institutional ethics committee and comply with Spanish and European Community regulations for use of laboratory animals.

Animals were actively sensitised as outlined previously [12]. Sensitised animals were randomly distributed into negative control (vehicle plus saline), positive control (vehicle plus antigen) and NAC-treated (NAC plus saline or antigen) groups. NAC (Zambon, Barcelona, Spain; 3 mmol·kg body weight^{-1}) in distilled water was administered orally by gavage as a single daily dose (at 09:00 h) from 7 days before challenge, with the last dose being given 1 h before challenge. The oral route was selected as usual in the clinical setting. The dose level and schedule were based on previous studies [5, 11]. Dexamethasone (5 mg·kg body weight^{-1} *i.p.*) was given in some experiments 24 h before challenge as a reference drug.

Animals were anaesthetised and instrumented for measurement of lung resistance (*R*L) as outlined previously [12]. After stabilisation for 10 min, animals were challenged with inhaled ovalbumin (100 mg·mL^{-1} for 5 min) for measurement of the immediate bronchoconstrictor response and Evans blue extravasation into BALF. In other experiments, unanaesthetised rats were exposed to ovalbumin (1% in saline) or saline aerosol for 60 min and, 24 h later, airway reactivity was determined in anaesthetised animals from dose/response curves to 5-HT (6.25–100 μg·kg body weight^{-1} *i.v.*). The slope of the straight-line regression between peak *R*L and the common logarithm ($\log_{10}$) of the 5-HT dose, using the data from only the 6.25, 12.5 and 25 μg·kg body weight^{-1} doses, was calculated for each experimental group [13]. In a different set of animals, BALF was obtained before and at different times after challenge for determination of total and differential cell counts [13], TNF-α levels by a competitive enzyme immunoassay kit (Chemicon International, Temecula, CA, USA) and lipid peroxidation (Lipid Hydroperoxide Assay kit; Cayman Chemical, Ann Arbor, MI, USA).

GSH was measured in lung tissue homogenates using the GSH transferase method [14] and oxidised glutathione (GSSG) by high-performance liquid chromatography [15]. *N*-Ethylmaleimide, a thiol-quenching agent, was used to effectively prevent GSH oxidation [15]. The GSH/GSSG ratio was calculated as an index of the tissue redox state.

Determination of nuclear factor-κB-binding activity and inducible nitric oxide synthase, intercellular adhesion molecule-1 and mucin MUC5AC expression

Nuclear protein extracts were prepared from lung tissue [16]. Protein quantification was carried out using the BRADFORD [17] assay. Aliquots of nuclear extracts with equal amounts of protein (10 μg) were processed according to manufacturers′ instructions (DIG gel shift kit; Boehringer Mannheim, Mannheim, Germany, and Enzo Diagnostics, Inc., Farmingdale, NY, USA), and binding reactions started by addition of 30 fmol of double-stranded digoxigenin-labelled NF-κB oligonucleotide (sense-strand sequence 5′-AGTTGAGGGGACTTTCC-CAGGC-3′, GGGGACTTTCC being a κB-binding motif) from Promega Co. (Madison, WI, USA). Samples were analysed on a 6% nondenaturating polyacrylamide gel. After electrophoretic transfer to a nylon membrane (Hybond-N+; Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany), complexes were visualised using a chemiluminescence detection system. The intensity of the bands was quantified using the image analysis system AnalySIS® 3.0 (Soft Imaging System GmbH, Münster, Germany). In order to ascertain the specificity of the binding reaction, competition assays were performed in the presence of an 100-fold excess (*i.e.* 3,000 fmol) of unlabelled oligonucleotide.

Aliquots of supernatant containing cytosolic proteins (40 μg) were loaded on to a 10% polyacrylamide gel and submitted to sodium dodecyl sulphate polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes (Protran®, Schleicher & Schuell, Keene, NH, USA), which were incubated in blocking solution containing the primary antibodies, rabbit anti-iNOS polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; 1:1,000 dilution) or β-actin mouse monoclonal antibody (Sigma-Aldrich Corporation, St Louis, MO, USA; 1:10,000 dilution). The secondary antibodies (1:5,000 dilution), horseradish peroxidase-linked donkey antirabbit immunoglobulin G directed against iNOS and horseradish peroxidase-linked sheep anti-mouse immunoglobulin G directed against β-actin (Amersham Pharmacia Biotech Europe GmbH) were incubated in blocking solution for 2 h at 22°C. Immunoreactivity was detected with an enhanced chemiluminescence Western blot detection system (Amersham Pharmacia Biotech Europe GmbH). Films were scanned and densitometric analysis performed as above.

The ICAM-1 and mucin MUC5AC messenger ribonucleic acid (mRNA) transcripts were measured using a real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR). For ICAM-1, reverse transcription of RNA to generate complementary deoxyribonucleic acid (cDNA) was carried out using the SYBR® Green RT-PCR reagent kit (SYBR® Green PCR Master Mix; Applied Biosystems, Warrington, UK) as indicated by the manufacturer, and, for MUC5AC, with Taqman® RT reagents (PE Biosystems, Morrisville, NC, USA). The specificity of the PCR primers was tested under normal PCR conditions and the products of the reaction were electrophoresed into a 2.5% Nusieve® GTG® agarose (BioWhittaker Molecular Applications, Inc., Rockland, ME, USA) gel. A single band of the expected molecular size was observed for ICAM-1, MUC5AC and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Quantitative relative gene expression data were obtained using the comparative threshold cycle (C_t) method ($\Delta\Delta C_t$ method) as described by the manufacturer (PE-ABI PRISM 7700 Sequence Detection System; Applied Biosystems, Foster City, CA, USA) and reported previously [18]. For validation of the $\Delta\Delta C_t$ method, the C_t values for target and reference genes were measured at different input amounts of total RNA (25–3,000 ng), then, ΔC_t values (target *versus* reference) were plotted against log total RNA and the absolute value of the slope was found to be <0.1 (not shown), indicating a similar efficiency of the two systems. GAPDH was chosen as endogenous control gene. Total RNA was extracted using the Ultraclean total RNA tissue isolation kit (MO Bio, Solana Beach, CA, USA). The PCR primers for rat ICAM-1, MUC5AC, and GAPDH were designed using Primer Express (PE Biosystems) according to published cDNA sequences (table 1).

Data analysis

Data are presented as mean±SEM. Statistical analysis of results was carried out by analysis of variance followed by appropriate *post hoc* tests including Bonferroni correction and unpaired t-test. A p-value of <0.05 was considered significant.

Results

Lung oxidant status

The concentration of lipid hydroperoxides in BALF was significantly increased 2 h after antigen challenge (2.7±0.4

Table 1. – Primers and probes for real-time quantitative reverse transcriptase polymerase chain reaction

Gene	Primers/probes	Sequence	Product size bp	Genbank#
ICAM-1	Forward	5′-CAGACCCTGGAGATGGAGAAG-3′	150	NM012967
	Reverse	5′-AGTACTGTTCAGAAGCACCACCTGT-3′		
MUC5AC	Forward	5′-CTGCCACATGTTGGACTTGG-3′	102	U83139
	Reverse	5′-TTGGTATGGCTTCTCGAGGG-3′		
	TaqMan probe	5′-CATCACTATGTGCAGCCCAAGGCG-3′		
GAPDH	Forward	5′-CCTGGAGAAACCTGCCAAGTATG-3′	103	NM017008
	Reverse	5′-ACAACCTGGTCCTCAGTGTAGCC-3′		
	TaqMan probe	5′-CAAGAAGGTGGTGAAGCAGGCGGC-3′		

bp: base pairs; ICAM-1: intercellular adhesion molecule-1; GAPDH: glyceraldehyde 3-phosphate dehydrogenase. #: accession numbers (Genbank, Bethesda, MD, USA).

nmol·mL^{-1}, n=5; $p<0.05$) compared with the saline control (0.8±0.3 nmol·mL^{-1}, n=5) and these augmented levels were attenuated in NAC-treated animals (1.3±0.3 nmol·mL^{-1}, n=5; $p<0.05$ *versus* positive control).

Lung GSH levels showed a tendency to decrease 2 h after antigen exposure but this failed to reach significance (fig. 1). In contrast, lung GSSG level was significantly increased 2 h after challenge compared with control; this increase was reduced in NAC-treated rats. The GSH/GSSG ratio, an index of the tissue redox state, was decreased 2 h after antigen challenge and this change was also reversed by NAC. No significant changes in lung GSH and GSSG were observed 24 h after challenge (data not shown).

Allergen-induced nuclear factor-κB-binding activity and inducible nitric oxide synthase, intercellular adhesion molecule-1, tumour necrosis factor-α and mucin MUC5AC expression

NF-κB DNA-binding activity was increased 2 h after allergen challenge, declining at 4 and 24 h after challenge. NAC abolished the early NF-κB activation observed 2 and 4 h after antigen challenge (fig. 2) with similar results for dexamethasone. Allergen exposure generated intense iNOS protein (fig. 2) and ICAM-1 mRNA (fig. 3) signals 4 h after challenge. No increased expression of ICAM-1 was found 24 h after challenge (data not shown). Treatment with NAC or dexamethasone abolished antigen-induced iNOS and ICAM-1 expression.

Two hours after antigen challenge, there was a marked increase in BALF TNF-α concentration, which then declined towards prechallenge levels (fig. 4). Treatment with NAC depressed this early antigen-induced augmentation of TNF-α, and dexamethasone produced similar effects.

Two hours after challenge, there was a slight increase in MUC5AC expression in the antigen-exposed group of untreated animals, and a significant two-fold increase was observed at 4 h; this enhancement of expression was abolished in the groups treated with NAC or dexamethasone (fig. 5).

Antigen-induced bronchospasm, airways hyperresponsiveness, eosinophilia and extravasation

Challenge of sensitised untreated animals with antigen aerosol provoked an immediate rise in *R*L (151±16%, n=5) accompanied by extravasation (the Evans blue concentration increased from 617±96 ng·mL^{-1} in saline-challenged rats to 1,335±139 ng·mL^{-1}, n=5 in each group; $p<0.05$). Treatment with NAC did not significantly reduce antigen-induced bronchoconstriction (103±39%, n=6) but markedly inhibited extravasation (709±54 ng·mL^{-1}, n=5; $p<0.05$ *versus* antigen-challenged). NAC alone altered neither *R*L nor extravasation in saline-challenged rats nor introduced significant changes in arterial blood pressure (data not shown).

Hyperresponsiveness to 5-HT was observed in antigen-challenged rats compared with controls (fig. 6a), whereas decreased responsiveness (*i.e.* an antihyperresponsiveness effect) was noticed in NAC- and dexamethasone-treated rats. There were no significant differences in the slopes of the dose/response curves to 5-HT between groups (fig. 6a). NAC alone did not modify airways responsiveness since the 5-HT dose/response curve obtained in saline-challenged rats did not differ between treated and untreated rats (data not shown).

Antigen-challenged rats showed a marked increase in the total number of BALF cells at 24 h (0.64±0.04×10^6 cells·mL^{-1} (n=8) *versus* 0.21±0.02×10^6 cells·mL^{-1} (n=8) in saline group; $p<0.05$). The total cell count at 24 h in antigen-challenged rats was significantly diminished by NAC (0.41±0.06×10^6 cells·mL^{-1} (n=14) *versus* saline control; $p<0.05$) or dexamethasone (data not shown) treatment. The differential cell count showed an increase in eosinophil number as well as in other cell types in BALF from antigen-challenged rats compared with that from saline-exposed animals (fig. 6b). The increase in eosinophil number at 24 h was reduced by NAC, whereas no significant inhibitory effect was produced by this agent on the remaining cell types, *i.e.* neutrophils, lymphocytes and macrophages. Dexamethasone markedly decreased numbers of all cell types except macrophages. A small increase in eosinophil number was noticed 4 h after antigen challenge and a tendency to lower numbers was observed in the drug-treated groups (fig. 6c). The total and differential cell counts observed in saline-challenged rats treated with NAC or dexamethasone did not differ from counts obtained in saline-challenged untreated rats (data not shown).

Discussion

NAC is a thiol compound endowed with antioxidant properties [1] that reduces the lung damage produced by oxidant stress in different experimental models and exerts beneficial effects in pulmonary diseases in which oxidant stress appears pathogenetically relevant [2]. In the present study, preliminary observations with NAC [10] have been extended to show the antioxidative, anti-inflammatory and antihyperresponsiveness effects of oral treatment with NAC (3 mmol·kg body weight^{-1}·day^{-1} for 1 week before challenge) in an established experimental model of allergic asthma [12].

Allergen challenge of the peripheral airways in atopic asthmatics has been demonstrated to produce, immediately, significant amounts of reactive oxygen species released locally from eosinophils and other inflammatory cells [1]. Therefore, the present study looked first for experimental evidence of

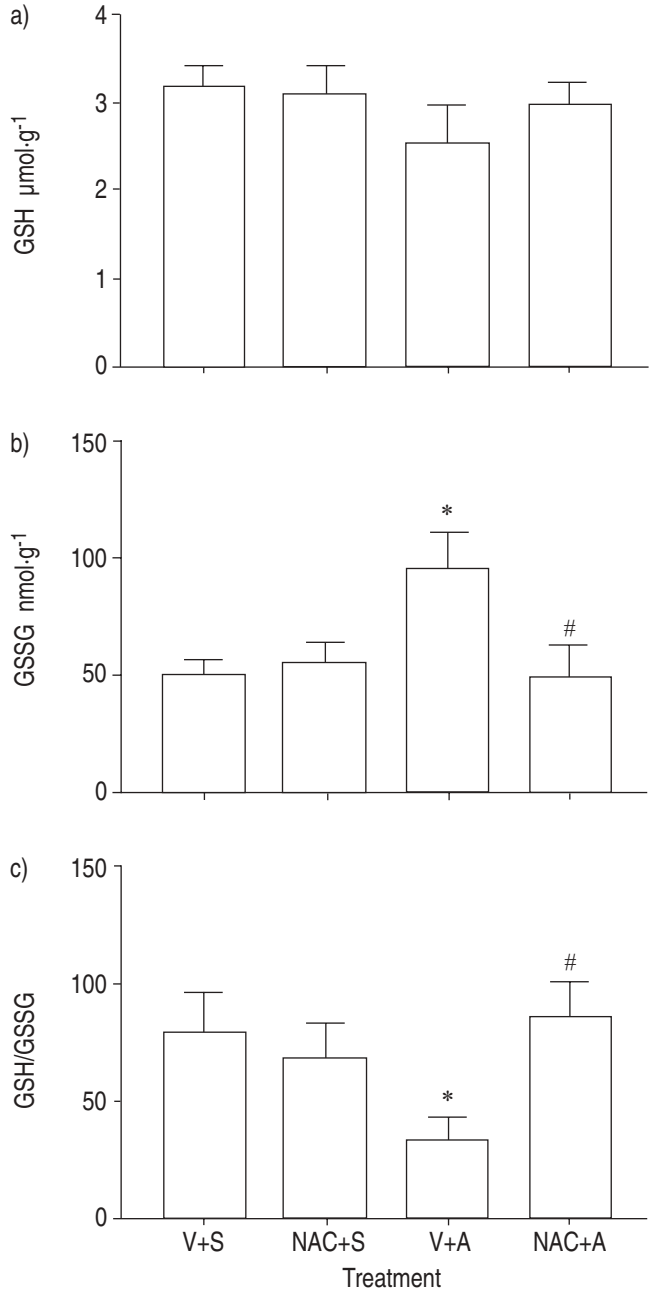

Fig. 1. – Effects of *N*-acetylcysteine (NAC) on a) reduced glutathione level (GSH); b) oxidised glutathione level (GSSG); and c) GSH/GSSG ratio in lung tissue. Animals were challenged with saline (negative control: vehicle plus saline (V+S)) or antigen (positive control: vehicle plus antigen (V+A)) or pretreated with NAC and then challenged (NAC plus saline (NAC+S) and NAC plus antigen (NAC+A)). The observations were made 2 h after challenge with antigen aerosol. Data are presented as mean±SEM of eight independent experiments for each group except for NAC plus saline (n=5). *: $p<0.05$ *versus* negative control; #: $p<0.05$ *versus* positive control.

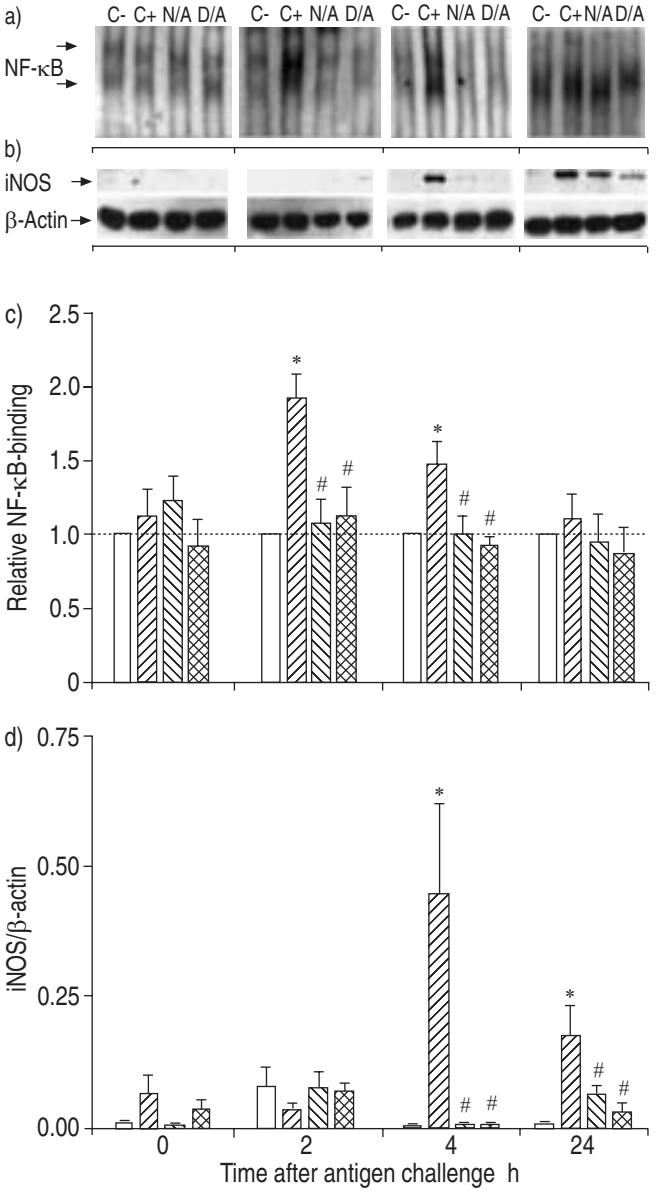

Fig. 2. – Effects of *N*-acetylcysteine (NAC) and dexamethasone (DEX) on allergen-induced changes in nuclear transcription factor (NF)-κB-binding activity and inducible nitric oxide synthase (iNOS) concentration. a) Electrophoretic gel mobility shift assay showing NF-κB-binding activity of rat lung cell nuclear proteins, b) Western blot for iNOS and β-actin; c) densitometric band shift data expressed relative to negative controls (C-; vehicle plus saline; □), which were taken as unity (·····); and d) densitometric Western blot data (▨: vehicle plus antigen (positive control (C+)). ▧: NAC plus antigen (N/A); ■: DEX plus antigen (D/A)). Densitometric data are presented as mean±SEM (n=6 independent experiments for each group (n=3 for Western blot negative control)). Note the increase in NF-κB binding activity 2 h after allergen exposure, returning towards baseline levels at 24 h, and the subsequent increase in iNOS concentration, peaking 4 h after allergen exposure and declining afterwards, but remaining above baseline levels at 24 h. *: $p<0.05$ *versus* negative control; #: $p<0.05$ *versus* positive control.

oxidative stress in this experimental model using two different markers, BALF levels of lipid hydroperoxides and lung GSH/GSSG ratio. Two hours after antigen challenge, there was an increase in lipid peroxidation levels and a decreased GSH/GSSG ratio, confirming the existence of oxidative stress. The decrease in GSH/GSSG ratio found in this study was due to an approximately two-fold significant increase in GSSG concentration accompanied by a tendency to lower GSH levels. An increase in GSSG and decrease in GSH level in epithelial lining fluid early after antigen challenge has been reported recently in asthmatics [19]. Oral treatment with NAC was efficient at attenuating the augmented lipid peroxidation and GSSG levels, and reversing the decreased GSH/GSSG ratio, confirming its antioxidative properties in this animal model.

Since the presence of oxidative stress was demonstrated for this model of allergic asthma, activation of a number of

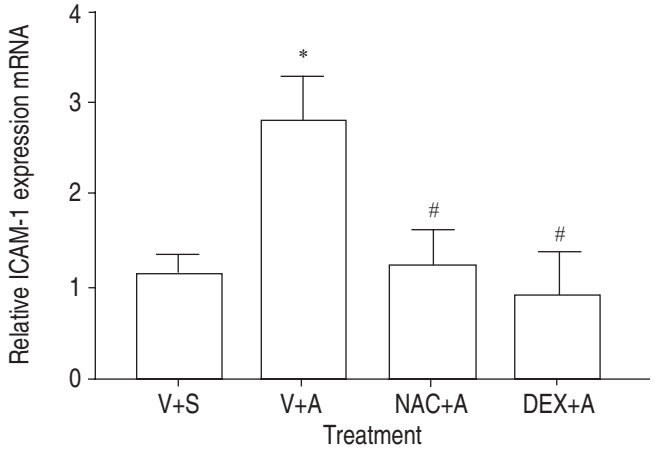

Fig. 3. – Relative quantification of intercellular adhesion molecule-1 (ICAM-1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) messenger ribonucleic acid (mRNA) 4 h after antigen aerosol challenge by real-time quantitative reverse transcriptase polymerase chain reaction using the comparative threshold cycle (C_t) method ($\Delta\Delta C_t$ method). Animals were challenged with saline (negative control: vehicle plus saline (V+S)) or antigen (positive control: vehicle plus antigen (V+A)) or pretreated with *N*-acetylcysteine (NAC) or dexamethasone (DEX) and then challenged (NAC plus antigen (NAC+A) or DEX plus antigen (DEX+A)) (n=5 for all groups). The C_t values for GAPDH were similar in the different samples confirming the value of this housekeeping gene as endogenous control. Data are presented as mean±SEM $2^{-\Delta\Delta Ct}$ relative to GAPDH. *: $p<0.05$ *versus* negative control; #: $p<0.05$ *versus* positive control.

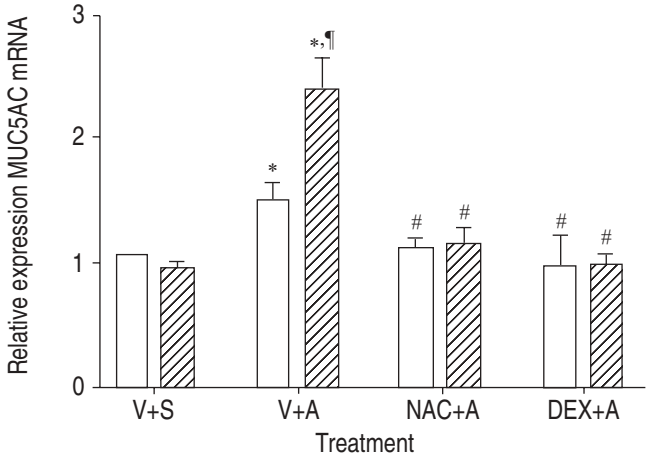

Fig. 5. – Relative quantification of mucin MUC5AC and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) messenger ribonucleic acid (mRNA) 2 (□) and 24 h (▨) after antigen aerosol challenge by real-time quantitative reverse transcriptase polymerase chain reaction using the comparative threshold cycle (C_t) method ($\Delta\Delta C_t$ method). Animals were challenged with saline (negative control: vehicle plus saline (V+S)) or antigen (positive control: vehicle plus antigen (V+A)) or pretreated with *N*-acetylcysteine (NAC) or dexamethasone (DEX) and then challenged (NAC plus antigen (NAC+A) or DEX plus antigen (DEX+A)) (n=3 for all groups). The C_t values for GAPDH were similar in the different samples confirming the value of this housekeeping gene as endogenous control. Data are presented as mean± SEM $2^{-\Delta\Delta Ct}$ relative to GAPDH. *: $p<0.05$ *versus* negative control; #: $p<0.05$ *versus* positive control; ¶: $p<0.05$ *versus* same group at 2 h.

inflammatory elements reported to be oxidant-sensitive, including transcription factors like NF-κB [20] and cytokines such as TNF-α [6], and expression of genes like iNOS [7], ICAM-1 [8] and MUC5AC [9] were sought. Furthermore, treatment with an antioxidant should attenuate these activated factors as well as prove beneficial against the typical features of experimental asthma such as airways hyperresponsiveness, eosinophilia and exudation.

NF-κB is considered a pivotal transcription factor in chronic inflammatory diseases and very sensitive to oxidants as well as other stimuli [20]. Augmented activation of NF-κB has been demonstrated in the airways and inflammatory cells

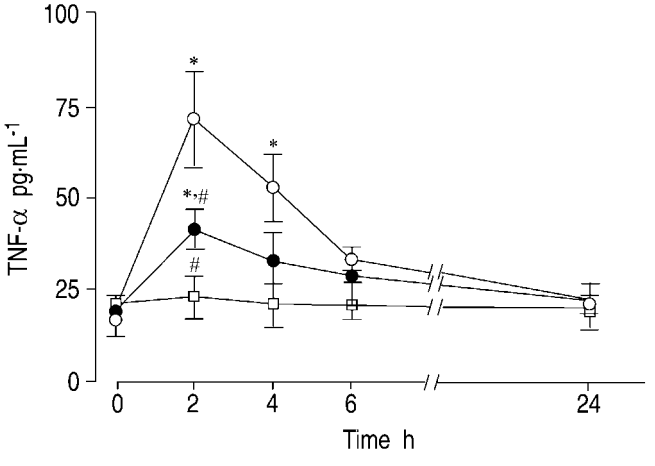

Fig. 4. – Effects of *N*-acetylcysteine (NAC; ●) and dexamethasone (□) on allergen-induced tumour necrosis factor (TNF)-α production in bronchoalveolar lavage fluid (○: vehicle plus antigen (positive control)). TNF-α levels were measured at several time points after aerosolised ovalbumin challenge in sensitised rats. Data are presented as mean±SEM (n=5). *: $p<0.05$ *versus* prechallenge values; #: $p<0.05$ *versus* positive control.

of asthmatic patients [21] as well as in experimental asthma [22, 23]. The early augmentation of NF-κB DNA-binding activity reported in lung nuclear extracts from sensitised challenged rats [22] was confirmed and this observation extended to show that the antioxidant NAC suppressed NF-κB activation in this asthma model. This finding is consistent with previous reports of the *in vivo* inhibitory activity of NAC on lipopolysaccharide-induced NF-κB activation in rat lung [5] and other experimental models [8]. The antioxidant properties of NAC found in the present study may contribute directly to its inhibitory effects on NF-κB activation. Alternatively, NF-κB activation may result from the release of TNF-α, which induces generation of reactive oxygen species [24] and has been found to be elevated in BALF from sensitised rats as early as 1 h after antigen challenge [25].

TNF-α is a pro-inflammatory cytokine that has been implicated in the pathogenesis of asthma and considered a potential target for therapeutic intervention. In the present study, it was confirmed that antigen exposure increased TNF-α levels in BALF 2 h after antigen challenge and that they then declined towards prechallenge values [25]. This increased TNF-α level was attenuated in NAC-treated animals, a finding consistent with the suggestion that GSH status regulates TNF-α production *in vivo* and with the inhibition by NAC of the increase in TNF-α observed in various studies [6, 8].

Enhanced iNOS levels were detected 4 h after antigen challenge in sensitised rats and returned towards baseline values 24 h after allergen exposure. The demonstration that NF-κB-binding was markedly increased 2 h after challenge, preceding the increase in iNOS mRNA [22] and protein (present study) expression, supports the involvement of NF-κB in this process and is consistent with the presence of NF-κB binding sites in the murine iNOS gene. Treatment with NAC suppressed this augmented iNOS expression in the present study, a finding that is in keeping with the demonstrated capacity of NAC to inhibit iNOS in other models [8].

The ICAM-1 gene contains NF-κB-binding sites and its

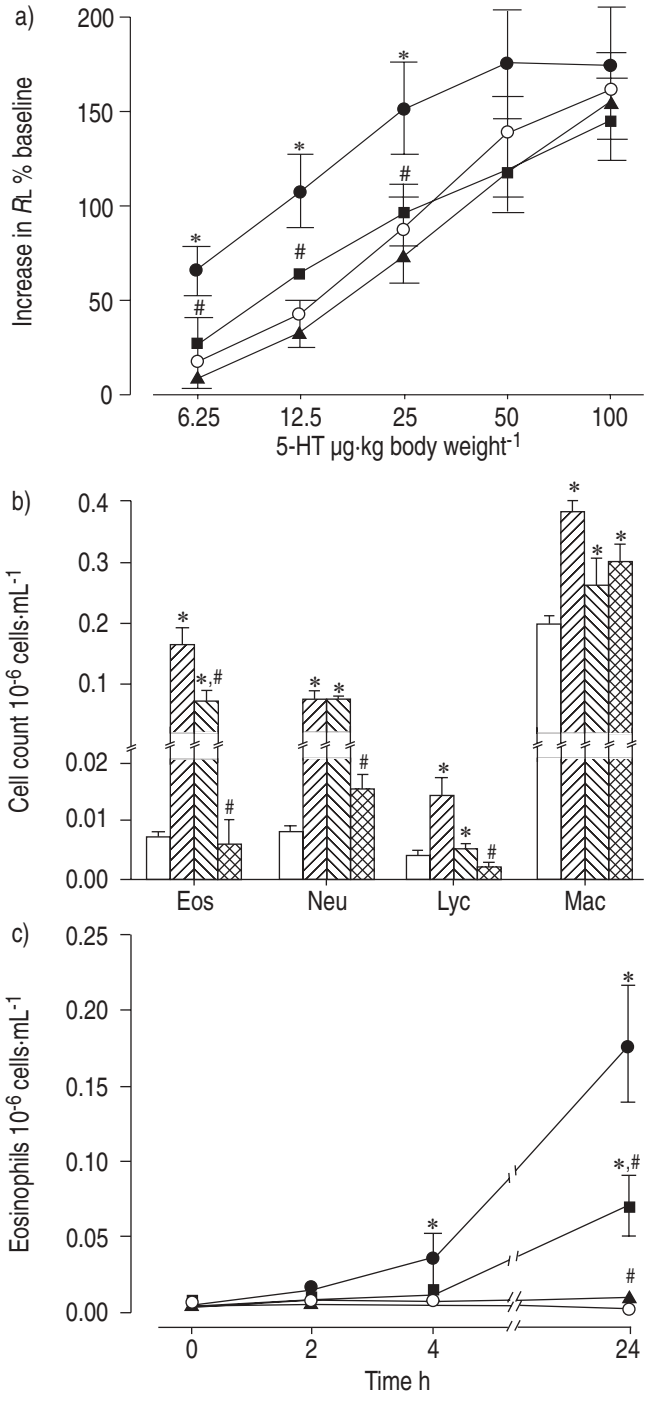

Fig. 6. – Effects of *N*-acetylcysteine (NAC) on: a) airways hyperresponsiveness; b) differential cell counts in bronchoalveolar lavage fluid (BALF); and c) time course of eosinophil number in BALF (○, □: vehicle plus saline (negative control); ●, ▨: vehicle plus antigen (positive control); ■, ▩: NAC plus antigen; ▲, ■: dexamethasone (DEX) plus antigen). a) Dose/response curves for 5-hydroxytryptamine (5-HT; intravenous) in sensitised rats after 24-h exposure to saline or antigen (ovalbumin 1%, 60 min) aerosol and treatment with NAC or DEX. Baseline lung resistance (R_L) values were 150±7 (negative control), 145±8 (positive control), 154±7 (NAC plus antigen) and 159±6 (DEX plus antigen) $cmH_2O \cdot L^{-1} \cdot s^{-1}$. The slopes of the dose/response curves were 183±39 (negative control), 213±74 (positive control), 181±42 (NAC plus antigen) and 158±19 (DEX plus antigen) $cmH_2O \cdot L^{-1} \cdot s^{-1} \cdot \log_{10}$ 5-HT dose^{-1}. Data are presented as mean±SEM of: a) 11–14; b) 11–12; and c) 4–6 animals per group. Eos: eosinophil; Neu: neutrophil; Lym: lymphocyte; Mac: macrophage. *: $p<0.05$ *versus* negative control; #: $p<0.05$ *versus* positive control.

expression is oxidant-sensitive [26]. The expression of airway and endothelial ICAM-1 is enhanced by TNF-α and other inflammatory cytokines [27]. Therefore, various elements may contribute to the enhanced expression found in the present study, and the inhibition found for NAC would be consistent with previous reports [8].

Mucus overproduction is often observed in airway inflammation and contributes to airway obstruction in asthma. Recent work indicates that oxidative stress stimulates mucin synthesis in airways, particularly synthesis of MUC5AC [9]. The early increase in MUC5AC expression, preceding eosinophil infiltration, has been demonstrated in a guinea-pig model of allergic asthma [28]. In keeping with these results, enhancement of MUC5AC expression at 2 h, when no increase in eosinophil number in BALF was yet detectable, and a further increase at 4 h after antigen challenge were found. Treatment with NAC blocked this early expression of MUC5AC. These results confirm that oxidative stress appears important in the excessive production of mucin in airways, and antioxidants are effective at suppressing the enhanced expression of mucin genes in experimental asthma.

Consequential to these inhibitory effects of antioxidant treatment on transcription factors, inflammatory cytokines and genes, there should be experimental evidence of beneficial effects of NAC on characteristic features of allergic asthma. NAC failed to reduce the increase in R_L produced immediately after antigen challenge in the present study [10]. Since antigen-induced bronchoconstriction is brought about mainly *via* the release of mediators from mast cells in airways, this result indicates that NAC is not effective at inhibiting this process, confirming the results of *in vitro* studies [29]. This would also be in agreement with an unimportant contribution of oxidant species to the immediate bronchoconstrictor response to antigen. Indeed, even relatively high concentrations of hydrogen peroxide produce only minor and transient contraction of isolated airways [30].

By contrast, oxygen radicals appear to be involved in the plasma extravasation elicited by antigen challenge in rats, and NAC abolished the airway extravasation following antigen provocation. This result is in agreement with recent reports showing antiexudative properties for NAC and other antioxidants [10, 13].

The presence of airways hyperresponsiveness and eosinophilia as a late reaction 24 h after antigen challenge of sensitised animals is well established in the literature [12, 13]. NAC was effective at reducing both airways hyperresponsiveness to 5-HT and the elevated BALF eosinophil numbers in the present study [10]. Several lines of evidence suggest that the production of oxygen radicals is implicated in airway responses to allergen. Thus, the antigen-induced hyperresponsiveness was found to correlate significantly with increases in oxygen radical release from bronchoalveolar lavage cells in sensitised dogs [30]. The oxidant-sensitive transcription factor NF-κB appears relevant to eosinophilia in allergic asthma [23]. Also, cell trafficking into inflammatory sites depends on the sequential expression of cell adhesion molecules which are modulated by oxidant species; in particular, ICAM-1 is important for induction of airways hyperresponsiveness *in vivo* as well as eosinophil migration into inflamed lung [27]. Therefore, the reduced airways hyperresponsiveness and eosinophilia produced by NAC may also be related to its antioxidant properties. In keeping with these results, it has been reported that antioxidants are endowed with antihyperresponsiveness and anti-inflammatory properties in experimental models of pulmonary inflammation [8, 13].

In summary, oral administration of *N*-acetylcysteine before antigen exposure in actively sensitised rats, a widely used experimental model of asthma, resulted in: 1) attenuation of antigen-induced augmented lipid peroxidation and altered

glutathione status; 2) suppression of the nuclear factor-κB activation, elevated tumour necrosis factor-α levels and enhanced inducible nitric oxide synthase, intercellular adhesion molecule-1 and mucin MUC5AC expression that follow allergen exposure; and 3) a marked decrease in airways hyperresponsiveness, bronchoalveolar lavage fluid eosinophil number and exudation after antigen challenge. These results confirm that oxidative stress may contribute to the pathogenesis of asthma. The potential therapeutic value of antioxidants including *N*-acetylcysteine awaits support from controlled clinical trials.

References

1. Henricks PAJ, Nijkamp FP. Reactive oxygen species as mediators in asthma. *Pulm Pharmacol Ther* 2001; 14: 409–421.
2. Cotgreave IA. *N*-Acetylcysteine: pharmacological considerations and experimental and clinical applications. *Adv Pharmacol* 1997; 38: 205–227.
3. Millman M, Millman FM, Goldstein IM, Mercandetti AJ. Use of acetylcysteine in bronchial asthma–another look. *Ann Allergy* 1985; 54: 294–296.
4. Bylin G, Hedenstierna G, Lagerstrand L, Wagner PD. No influence of acetylcysteine on gas exchange and spirometry in chronic asthma. *Eur J Respir Dis* 1987; 71: 102–107.
5. Blackwell TS, Blackwell TR, Holden EP, Christman BW, Christman JW. *In vivo* antioxidant treatment suppresses nuclear factor-κB activation and neutrophilic lung inflammation. *J Immunol* 1996; 157: 1630–1637.
6. Peristeris P, Clark BD, Gatti S, *et al.* *N*-Acetylcysteine and glutathione as inhibitors of tumor necrosis factor production. *Cell Immunol* 1992; 140: 390–399.
7. Pahan K, Sheikh FG, Namboodiri AMS, Singh I. *N*-Acetylcysteine inhibits induction of NO production by endotoxin or cytokine stimulated rat peritoneal macrophages, C_6 glial cells and astrocytes. *Free Radic Biol Med* 1998; 24: 39–48.
8. Cuzzocrea S, Mazzon E, Dugo L, *et al.* Protective effects of *N*-acetylcysteine on lung injury and red blood cell modification induced by carrageenan in the rat. *FASEB J* 2001; 15: 1187–1200.
9. Takeyama K, Dabbagh K, Shim JJ, Dao-Pick T, Ueki IF, Nadel JA. Oxidative stress causes mucin synthesis *via* transactivation of epidermal growth factor receptor: role of neutrophils. *J Immunol* 2000; 164: 1546–1552.
10. Blesa S, Cortijo J, Martinez-Losa M, *et al.* Effectiveness of oral *N*-acetylcysteine in a rat experimental model of asthma. *Pharmacol Res* 2002; 45: 135–140.
11. Cortijo J, Cerdá-Nicolás M, Serrano A, *et al.* Attenuation by oral *N*-acetylcysteine of bleomycin-induced lung injury in rats. *Eur Respir J* 2001; 17: 1228–1235.
12. Cortijo J, Blesa S, Martinez-Losa M, *et al.* Effects of taurine on pulmonary responses to antigen in sensitized Brown-Norway rats. *Eur J Pharmacol* 2001; 431: 111–117.
13. Inman MD, Ellis R, Wattie J, Denburg JA, O′Byrne PM. Allergen-induced increase in airway responsiveness, airway eosinophilia, and bone-marrow eosinophil progenitors in mice. *Am J Respir Cell Mol Biol* 1999; 21: 473–479.
14. Brigelius R, Muckel C, Akerboom TPM, Sies H. Identification and quantitation of glutathione in hepatic protein mixed disulfides and its relationship to glutathione disulfide. *Biochem Pharmacol* 1983; 32: 2529–2534.
15. Asensi M, Sastre J, Pallardó FV, García de la Asunción J, Estrela JM, Viña J. A high-performance liquid chromatography method for measurement of oxidized glutathione in biological samples. *Anal Biochem* 1994; 217: 323–328.
16. Deryckere F, Gannon F. A one-hour minipreparation technique for extraction of DNA-binding proteins from animal tissues. *Biotechniques* 1994; 16: 405.
17. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; 72: 248–254.
18. Dasí FJ, Ortiz JL, Cortijo J, Morcillo EJ. Histamine up-regulates phosphodiesterase 4 activity and reduces prostaglandin E_2-inhibitory effects in human neutrophils. *Inflamm Res* 2000; 49: 600–609.
19. Comhair SA, Bhathena PR, Dweik RA, Kavuru M, Erzurum SC. Rapid loss of superoxide dismutase activity during antigen-induced asthmatic response. *Lancet* 2000; 355: 624.
20. Barnes PJ, Karin M. Nuclear factor-κB – a pivotal transcription factor in chronic inflammatory diseases. *N Engl J Med* 1997; 336: 1066–1071.
21. Hart LA, Krishnan VL, Adcock IM, Barnes PJ, Chung KF. Activation and localization of transcription factor, nuclear factor-κB, in asthma. *Am J Respir Crit Care Med* 1998; 158: 1585–1592.
22. Liu SF, Haddad E-B, Adcock I, *et al.* Inducible nitric oxide synthase after sensitization and allergen challenge of Brown Norway rat lung. *Br J Pharmacol* 1997; 121: 1241–1246.
23. Yang L, Cohn L, Zhang D-H, Homer R, Ray A, Ray P. Essential role of nuclear factor κB in the induction of eosinophilia in allergic airway inflammation. *J Exp Med* 1998; 188: 1739–1750.
24. Ko S, Kwok TT, Fung KP, Choy YM, Lee CY, Kong SK. Tumour necrosis factor induced an early release of superoxide and a late mitochondrial membrane depolarization in L929 cells. Increase in the production of superoxide is not sufficient to mimic the action of TNF. *Biol Signals Recept* 2001; 10: 326–335.
25. Escott KJ, Belvisi MG, Birrell MA, Webber SE, Foster ML, Sargent CA. Effect of the p38 kinase inhibitor, SB 203580, on allergic airway inflammation in the rat. *Br J Pharmacol* 2000; 131: 173–176.
26. van de Stolpe A, van der Saag PT. Intercellular adhesion molecule-1. *J Mol Med* 1996; 74: 13–33.
27. Wegner CD, Gundel RH, Reilly P, Haynes N, Letts LG, Rothlein R. Intercellular adhesion molecule-1 (ICAM-1) in the pathogenesis of asthma. *Science* 1990; 247: 456–459.
28. Li Y, Martin LD, Minnicozzi M, *et al.* Enhanced expression of mucin genes in a guinea pig model of allergic asthma. *Am J Respir Cell Mol Biol* 2001; 25: 644–651.
29. Hong XJ, Francker A, Diamant B. Effects of *N*-acetylcysteine on histamine release by sodium fluoride and compound 48/80 from isolated rat mast cells. *Int Arch Allergy Appl Immunol* 1991; 96: 338–343.
30. Stevens WHM, Inman MD, Wattie J, O′Byrne PM. Allergen-induced oxygen radical release from bronchoalveolar lavage cells and airway hyperresponsiveness in dogs. *Am J Respir Crit Care Med* 1995; 151: 1526–1531.